Faster Evaluation of Induced Floral Sterility in Transgenic Early Flowering Poplar

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Abstract

A major concern over the use of transgenic trees is the potential for transgene dispersal through pollen and seeds. The incorporation of sterility inducing genes into transgenic lines of trees has been proposed to reduce or even avoid gene flow of transgenes into non-transgenic interbreeding species. The evaluation of strategies for the induction of sterility in transgenic forest tree species has been hindered by their long vegetative phases. In this study an early flowering 35S::*Leafy* poplar line was used for the faster evaluation of the sterility construct C-GPDHC::*Vst1*. The combination of two transgenic approaches, one to induce early flowering and a second for the induction of sterility, allowed evaluation of this sterility strategy two years after transformation. This is a very short period of time considering the long vegetative period of seven to twenty years common in forest tree species. This approach opens opportunities for the assessment of sterility mechanisms for this plant group.

Key words: sterility, biosafety, genetic engineering, transgenic trees, *Populus*, *leafy*, *stilbene synthase*, early flowering.

1. Introduction

The promising prospects offered by genetic engineering have promoted efforts to develop molecular breeding methods for woody plants. Genetic engineering of plants has some advantages in comparison to conventional breeding: (a) genes from virtually any organism can be introduced in the breeding process, thus broadening the range of genes available outside the current boundaries of the genus, (b) individual genotypes can be improved for one or a small number of well defined traits while preserving the rest of the genome, and (c) genetic engineering is the only method available which could allow accelerated breeding of forest tree species at rates comparable to that achieved with crop plants. This latter advantage is particularly important as breeding of forest trees has been hampered by the long time they require to enter into the reproductive phase.

However, despite of these advantages, genetic engineering has been the subject of considerable controversy, with concerns raised mainly from ecological and ethical arguments (reviewed in: HOENICKA and FLADUNG, 2006a; WALTER, 2004). A major concern considering the deployment of transgenic trees is the potential for transgene dispersal through pollen and seeds (ELLSTRAND, 2001; KUVSHINOV et al., 2001; MIKKELSEN et al., 1996). The incorporation of sterility genes into transgenic lines of trees has been proposed to reduce or even avoid gene flow of transgenes into interbreeding species (STRAUSS et al., 1995). The prevention of flower/inflorescence development in transgenic trees might be economically more advantageous since those sterile plants may use more of their resources for vegetative growth (BRUNNER et al., 1998; LEMMETYINEN, 2004; MOURADOV et al., 1998). However, it is not clear if sterile plants will in fact redirect their resources to produce more biomass.

Many strategies to induce sterility including the use of sterility gene constructs have successfully been tested in crop plants, e.g. use of dominant negative mutations (MITZUKAMI et al., 1996), gene suppression strategies such as antisense suppression, cosuppression, and RNA interference (SKINNER et al., 2003), by expression of cytotoxic genes, such as *barnase* (MARIANI et al., 1990) or using the gene for ribosome inactivating protein (PALMITER et al., 1987). Sterility conferring genes, however, need specific regulatory promoters (e.g. TA29 promoter from tobacco or PrMALE1 from *Pinus radiata*) to direct expression of genes in reproductive structures (KOLTUNOW et al., 1990; MARIANI et al., 1990; WALDEN et al., 1999, HOEFIG et al., 2003).

Few investigations have been reported on induction of sterility in trees using genetic modification. The first reports of successful floral sterility induction in a forest tree species were in early flowering silver birch (*Betula pendula* Roth) (LEMMETYINEN, 2004; LÄNNENPÄÄ et al., 2005). Gene constructs with the *barnase* gene and the BpMADS1 and BpFULL1 promoters were successfully used in birch with this aim (LEMMETYINEN, 2004; LÄNNENPÄÄ et al., 2005). Several approaches have been reported on the induction of sterility in poplar (MEILAN et al., 2001; FLADUNG and HOENICKA, 2004; HOENICKA and FLADUNG, 2003; SKINNER et al., 2003). However, the induction of sterility in transgenic poplar has not really been demonstrated adequately so far. The use of floral-specific promoters from tobacco (KOLTUNOW et al., 1990; WANG et al., 1993) and Brassica (HACKETT et al., 1992) to direct the expression of a ribosome inactivating protein (DTA) (PALMITER et al., 1987) or an extracellular ribonuclease (BEALS and GOLDBERG, 1997; HARTLEY, 1988) resulted in significantly decreased vegetative growth in poplar (MEILAN et al., 2001; SKINNER et al., 2000). HOEFIG et al. (2006) suggested to use the *Vitis vinifera stilbene synthase* gene (*sts*) under transcriptional control of a *Pinus radiata* male cone specific promoter to induce male sterility in *Pinus radiata*. The concept was suc-

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cessfully used to abolish pollen formation in the model species tobacco, however transgenic *Pinus radiata* plants need to grow for 5–7 years before the effect of this gene on pollen production in pine can be assessed (HOEFIG et al., 2006). The development of sterile transgenic trees should also consider genomic and transgene stability (HOENICKA and FLADUNG, 2006b). There are reports of unexpected variation in transgene expression levels in many plant species and also in aspen (FLADUNG, 1999; KUMAR and FLADUNG, 2001). Therefore, the stable integration of foreign genes in the genome of forest trees, and their subsequent stable expression during growth and development over long periods of time and under the influence of natural environmental conditions, are essential for the use of transgenic trees in forest tree breeding programs.

The most important drawback for studying induction of sterility in forest tree species is their long juvenile period. Chemical methods (using growth retardants, phytohormones etc), physical methods (vascular restriction, root growth control) and genetic transformation with early flowering gene constructs have been used in order to induce early flowering in trees (reviewed in MEILAN, 1997). Early flowering has been induced in poplar species using genetic transformation with the 35S::*Leafy* (WEIGEL and NILSSON, 1995), 35S::*rolC* (FLADUNG et al., 2003), 35S::*FT* (*FT*: *Flowering Locus T*) and 35S::*PtFT* (*FT* ortholog from *Populus trichocarpa*) constructs (BÖHLENIUS et al., 2006). Constitutive expression of *Leafy* and *FT* genes has allowed to accelerate flowering and even to obtain progeny in citrus trees (*Poncirus trifoliate* L. Raf.) (PENA et al., 2001; ENDO et al., 2005).

The first commercial plantations of GM Poplar (LIDA et al., 2003) and Papaya (GONSALVES, 1998; CHIANG et al., 2001) have been reported in China and the US, respectively. The deployment of transgenic forest trees is expected to increase in the next years. Considering the importance of tree species to the environment and global climate, transgenic trees should be thoroughly evaluated with respect to their biosafety and in order to avoid or mitigate potential risks to the environment and human health.

As mentioned before, first reports on poplar lines transformed with sterility constructs showed a decreased vegetative growth (MEILAN et al., 2001; SKINNER et al., 2000). The evaluation of new strategies for the induction of sterility is therefore very important. In this study, a gene construct consisting of the promoter of the cytosolic glycerin-3-phophate dehydrogenase C gene (CGPDH-C) from *Cuphea lanceolata* and the *stilbene synthase* gene (Vst1) from *Vitis vinifera* were tested in poplar. The overexpression of the *stilbene synthase* gene, reportedly causing male sterility in tobacco, seems to promote a competition between the stilbene synthase and the endogenous chalcone synthase for the substrates 4-coumaroyl CoA and malonyl CoA. (FISHER et al., 1997). The CGPDH-C promoter was found to be active only in anthers of *Brassica napus* and *Cuphea lanceolata* (HAUSMANN and TÖPFER, 1999). The generation of transgenic lines of poplar expressing both the early flowering 35S::*Leafy* and the sterility gene construct C-GPDHC::*Vst1*, allowed us to confirm within a short period of time the successful induction of sterility in poplar lines. This is an important contribution for the evaluation of possible risks derived from transgenic trees prior to their broad commercialisation.

2. Material and Methods

Preparation of transformation vectors and Agrobacterium strains

The induction of early flowering was achieved using the plasmid pDW 1151 (kindly provided by DETLEF WEIGEL, Max Planck Institute for Developmental Biology, Tuebingen) for transformation. This plasmid contained the *Leafy* gene (WEIGEL and NILSSON, 1995) from *Arabidopsis thaliana* under the control of the 35S promoter from the cauliflower mosaic virus (GUILLEY et al., 1982). Sterility was induced using the *stilbene synthase* gene (*Vst1*) (HAIN et al., 1993) from *Vitis vinifera* controlled by the promoter of the *cytosolic glycerin-3-phosphate dehydrogenase C* gene (C-GPDHC) from *Cuphea lanceolata* (HAUSMANN and TÖPFER, 1999).

The C-GPDHC::*Vst1* construct was obtained by PCR amplification of the *Vst1* coding sequence from plasmid pGB3 (kindly provided by D. Becker, Hamburg University with permission of R. Hain, Bayer Crop Science, Monheim) and insertion of the PCR product into the pGemTeasy TA-cloning vector. From there it was excised with *Eco*RI and inserted into the *Eco*RI site of pGreen II (ROGER et al., 2000; http://www.pgreen.ac.uk/a_pls_fr.htm). The resulting clones were checked for the orientation of the insert by sequencing and the C-GPDHC promoter was subsequently cloned from the vector PGpromC4 (kindly provided by L. Hausmann, Federal Centre for Breeding Research on Cultivated Plants, Siebeldingen) by excision with *Sca*I and *BamH*I, blunting with T4-polymerase and insertion into the *Sma*I site of the vector carrying the *Vst1* gene.

The restriction sites for *Stu*I and *Hpa*I located near to the right and left border sequences were used for the incorporation of marker cassettes for 35S::*nptII* (for kanamycin resistance) and 35S::*aphIII* (for hygromicin resistance) respectively. The presence of the hygromycin marker gene in the transformation vector allowed a second transformation of early flowering kanamycin-resistant 35S::*Leafy* lines. Transformation vectors were introduced into the *Agrobacterium* strain EHA105 (HOOD et al., 1993) using the freeze-thaw method (TZFIRA et al., 1997).

Plant material, culture and genetic transformation

In vitro cultures of two male clones (*Populus tremula* L. x *P. tremuloides* Michx., clone T89 and *Populus tremula* L., clone W52) were used for generation of the transgenic lines. Plants were grown on solid McCown Woody Plant Medium (WPM, Duchefa M0220) (LLOYD and MCCOWN, 1980) containing 2% Sacharose, 0.6%. Agar (Agar Agar, Serva, 11396). Genetic transformations were carried out using the *Agrobacterium*-mediated approach (FLADUNG et al., 1997). WPM medium for the regenera-

tion of transgenic plants was supplemented with 0.01% Pluronics F-68 (Sigma P-7061), thidiazuron (0.01 µM) and antibiotics, cefotaxime (500 mg/L) for *Agrobacteria* elimination and kanamycin (50 mg/L) or hygromycin (20 mg/L) for the selection of transgenic shoots.

Early flowering sterile plants were transferred to growth chambers (Weiss Technik) under the following culture conditions: light period: 16/8 (day/night), light intensity: 5–8 x 10^3 lux, lamps: Phillips TLM 140W/33RS, relative humidity: 89%, temperature: 22/19 °C. After a culture period of 6–18 months in the growth chambers transgenic plants were transferred and grown in a standard S1 greenhouse under natural daylight conditions.

Extraction of DNA and molecular analysis

DNA extraction was followed by a standard protocol adapted from DOYLE and DOYLE (1987), using 0.5–1.0 g leaf material and a modified extraction buffer [2% alkyltrimethylammonium bromide (ATMAB), 0.1 M Tris-HCl, 0.02 M disodium-EDTA (pH 8.0), 1.4 M NaCl, 1% PVP].

Standard PCR techniques were used to detect the transgenes. The following primer sequences were used to amplify trangenes: *Leafy* 5'-GTT GGT GAA CGG TAC GGT AT-3' and 5'-ACT AGA AAC GCA AGT CGT CG-3' and for the *Vst1* gene 5'-TGT AGA AAT GCC CGG TGC AGA-3' and 5'-CGC TAT GCA GCA CAA CGG TCT-3'. The PCR reaction used for all primers consisted of 94 °C / 2 min, followed by 40 cycles (94 °C / 1 min, 60 °C / 2 min, 72 °C / 2 min) and finally 72 °C / 5 min.

Southern hybridisations were carried out with 20 µg genomic DNA. 35S::*Leafy* DNA was digested with *BamH*I (only one restriction site present in the T-DNA) or *Bgl*II (two restriction sites are present in the T-DNA), C-GPDHC::*Vst1* DNA was digested with *Dra*I (only one restriction site present in the T-DNA). Subsequently, the DNA was separated using a 1.5% agarose gel and transferred onto a membrane by capillary transfer (Nylon membrane positively charged, Roche) in alkaline conditions. Prehybridization and hybridization were performed with the non-radiactive DIG (digoxigenine) system using DIG-dUTP-labelled gene probes (FLADUNG and AHUJA, 1995). DIG probes were prepared with a PCR amplification Kit (PCR DIG Probe Synthesis Kit, Roche) using the different plasmids with the respective primer pairs. Probe hybridization and chemiluminescent reaction were performed according to Roche instructions with some modifications (FLADUNG and AHUJA, 1995).

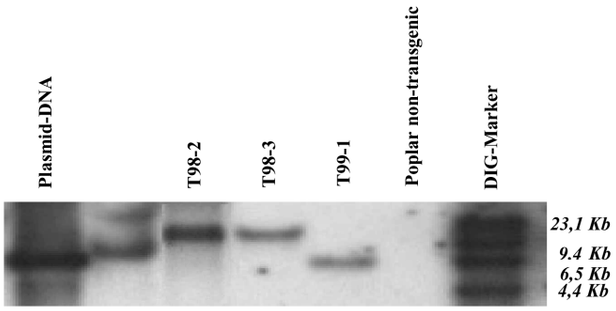

Figure 1. – Southern hybridisation of early flowering 35S::*Leafy* poplar transformed with sterility construct C-GPDHC::*Vst1*. 20 µg genomic DNA were digested with *Bgl*II, separated using gel electrophoresis, blotted on membrane and hybridized with DIG-dUTP-labelled probe (Approach for copy number detection).

Microscopic observation of anthers derived from transgenic plants

Anthers obtained form early flowering and early flowering-sterile flowers were stained with acetic orcein (1%) and observed under an optical microscope to confirm presence or absence of pollen grains.

3. Results

Early flowering 35S::Leafy poplar lines

Transgenic early flowering lines were obtained with the two different poplar clones used in this study (*Table 1*). Plants growing on selection medium, containing kanamycin, were studied using PCR and Southern hybridisation. Those transgenic lines containing only one complete copy of the 35S::*LFY* construct were selected. This selection criterion was based on information showing that the presence of more than one copy of the transgene in a transgenic plant can promote gene silencing (FLADUNG and KUMAR, 2002). The early flowering 35S::*Leafy* poplar shows a dwarf phenotype. In total, 7 transgenic lines containing one single copy of the transgene (single-copy line) were identified in order to select the best flowering lines for sterility gene transformations.

Transgenic lines were observed for about one year, and the degree of flowering varied considerably between the different single-copy lines selected. During that first year, four single-copy lines showed flowers and three did not. We could also observe that the single-copy transgenic line (T58-4) suddenly stopped flowering, and has not shown any flower in the following two years. Originally, this line was chosen for the generation of early flowering-sterile lines. Following this observation we

Table 1. – Genetic transformation of different poplar clones with the early flowering 35S::*Leafy* construct.

Poplar clone	Kanamycin-resistant lines	Transgenic lines Southern-tested
W52 ♂	71	6 (2*,**)
T89 ♂	30	6 (5*,**)

* Number of lines possessing one complete copy of gene construct. ** *In vitro* flowering observed in some lines.

Table 2. – Genetic transformation of early flowering 35S::*Leafy* hybrid poplar (lines T58-3) with sterility gene constructs.

Gene construct	Hygromycin-resistant lines	PCR-tested lines	Transgenic lines Southern-tested
C-GPDHC::*Vst1*	7	3	5 (3*,**)

* Number of lines possessing one copy of gene construct.
** *In vitro* flowering observed in some lines.

micropropagated one additional line, T58-3, for genetic transformation with the sterility construct. Plants derived from different transgenic lines were successfully transferred to the growth chamber and greenhouse.

Plants of line T58-3 grown in the greenhouse showed pollen grains (*Table 3*; *Figure 2*). Plants under *in vitro* and growth chamber conditions lacked pollen grains. Presence or absence of pollen grains in the greenhouse plants varied during the year, ranging from 0 to 100% flowers with pollen grains (*Table 3*). Pollen grains were found mainly in flowers during the spring months.

Early flowering-sterile poplar lines

Double transgenic lines, containing both the early flowering gene construct (35S::*Leafy*) and the sterility gene construct (C-GPDHC::*Vst1*), were grown on selection medium, containing hygromycin. Transgenic plants were studied using PCR and Southern hybridisation (*Table 2*, *Figure 1*). Based on these results, transgenic lines containing only one complete copy of the sterility construct were selected. Three different single-copy lines (lines T98-2, T98-3, T99-1) developed flowers under greenhouse conditions. All flowers hitherto obtained lacked pollen grains and had smaller anthers and longer stamens as compared to 35S::*Leafy* flowers (*Table 3*, *Fig. 2*).

Microscopic analysis of flowers

Anthers obtained from early flowering (T58-3) and early flowering-sterile flowers (T98-3, T98-2, T99-1) were stained with acetic orcein (1%). In 35S::*Leafy* poplar plants (T58-3) grown in the greenhouse flowers contained anthers with pollen grains (*Table 3*, *Figure 2*). Flowers from *in vitro* cultures, growth chamber plants and occasionally also greenhouse plants were devoid of pollen. In the last group very strong variations were found during the year. In all flowers from early flowering-sterile plants no pollen grains could be detected during one year of observation (*Table 3*, *Figure 2*).

4. Discussion

Poplars transformed with sterility genes were released to the field at the Oregon University/USA for the first time (CONFALONIERI et al., 2003). Information regarding activity of sterility constructs in those "normal flowering" transgenic lines is still not available, due to their long vegetative phase. In our study, the combination of two transgenic approaches, one to induce early flowering and a second one for the induction of sterility, allowed the evaluation of a new sterility strategy two years after transformation. This is a very short period of time considering the long vegetative phase of up to twenty years common in many forest tree species. This approach opens new opportunities for the assessment of mechanisms for transgene containment in transgenic forest tree species.

The evaluation of sterility constructs in forest tree species is hindered not only by the long vegetative periods but also by legal limitations to field release of transgenic plants. The use of early flowering poplar allows testing sterility strategies under greenhouse conditions, which are less limited by legal issues than field releases. The development of methods for the induction of early flowering in forest tree species might become an important tool for different studies with this group of plants. However, early flowering 35S::*Leafy* shows some handicaps, which should be avoided in future approaches. Developmental disturbances detected in poplar transformed with the 35S::*Leafy* construct, e.g. development of individual flowers instead of inflorescences, dwarf growth and occasional sterility, diminish the performance of this model system. The incomplete activation of flowering gene cascades by *Leafy* can explain this disturbances. An important asset of this plants is the induction of flowers all the year. However pollen grains were mainly observed in spring flowers. The variable activation of microsporogenesis was very puzzling. Environmental factors play possibly an important role herein. Natural daylight (variable and more intense) may be activating a circadian rhythm in poplar which induces genes involved in the microsporogenesis process. Temperature variations or even vernalization may be also playing an important role. Plants maintained under *in vitro* and growth chamber conditions were not subjected to such environmental factors and hence did not produce pollen grains. Molecular studies have identified a large number of genes that are expressed during pollen development (MA, 2005). However, the regulation of

Table 3. – Microscopic analysis of flowers from transgenic early flowering and early flowering-sterile lines or poplar.

Poplar clone (C) or transgenic line (TL) used for genetic transformation	Gene constructs	Flowering transgenic line	Number of plants	Number of flowers studied (number of flowers lacking pollen)
C: T89	35S::*Leafy*	T58-3	22	35 (15) *
TL: T58-3	C-GPDHC::*Vst1* 35S::*Leafy*	T98-3	5	4 (4)
		T98-2	4	9 (9)
		T99-1	6	14 (14)

* presence or absence of pollen grains in the T58-3 line varied during the year, oscillating from 0 to 100% flowers with pollen grains.

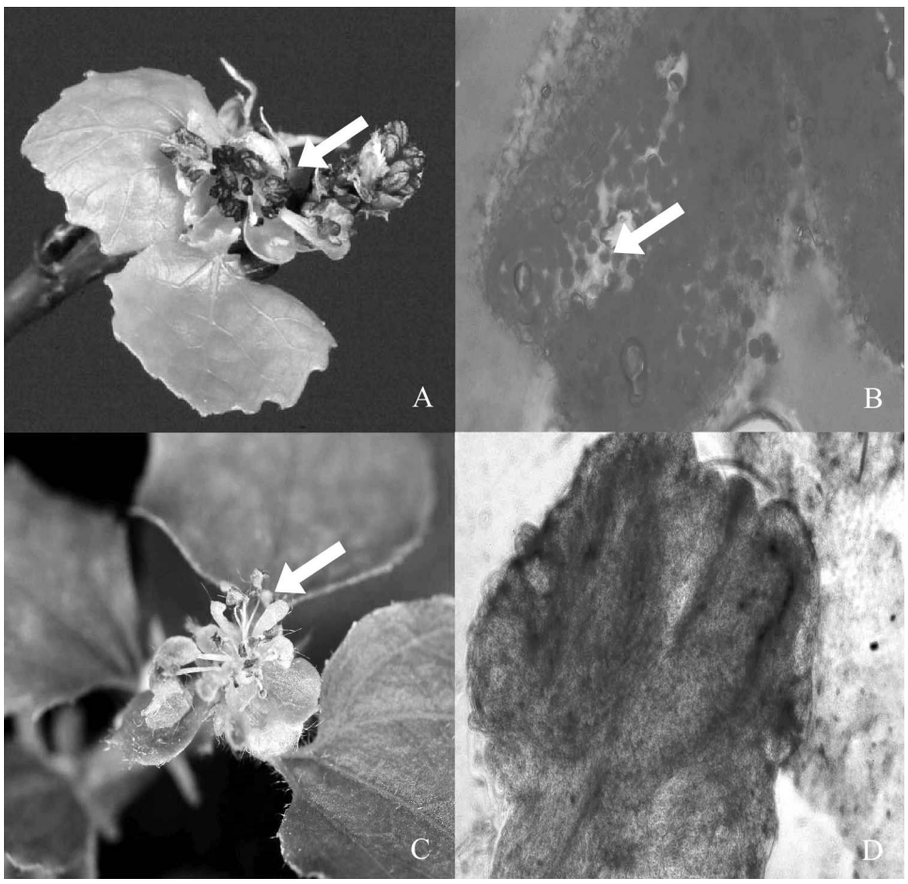

Figure 2. – Early flowering (T58-3) and early flowering-sterile poplar (T98-3). Arrows show flowers or pollen grains contained in anthers.
(A): Early flowering 35S::*Leafy*-poplar. (B) Detail of the flower and detail of anther containing pollen grains (stained with acetic orcein), (C): Early flowering-sterile poplar (transformed with 35S::*Leafy* and C-GPDHC::*Vst1*, (D): Detail of anther devoid of pollen grains (stained with acetic orcein).

microsporogenesis is not still well understood. Our results seems to indicate that *Leafy* is not involved in the regulation of microsporogenesis and only the adequate environmental signals are able to activate this process.

The induction of sterility was shown for poplar plants transformed with the construct C-GPDHC::*Vst1*, as only sterile flowers were obtained (T*able 3*). However, the system tested in this research, combining the 35S::*Leafy* and one sterility construct may require the evaluation of a higher number of flowers in order to eliminate false conclusions caused by the incidence of sterility in 35S::*Leafy* early flowering lines (*Table 3*). Despite of this handicap the 35S::*Leafy* could be successfully used for a faster evaluation of the C-GPDHC::*Vst1* sterility construct.

Several candidate genes, such as *BpMADS4*, *Rol C*, *Rol D* and *FT*, under control of the 35S promoter, were additionally tested for the promotion of early flowering and generation of early flowering and sterile lines of poplar (unpublished results). However, besides the constitutive expression of *Leafy* only the use of 35S::*FT* and 35S::*PtFT* (*FT* ortholog from *Populus trichocarpa*) allow a very fast flowering induction in poplar (BÖHLENIUS et al., 2006; HSU et al., 2006; HOENICKA and FLADUNG, unpublished). In the last years valuable progress has been achieved regarding the molecular mechanisms of flower induction (AHN et al., 2006; BÖHLENIUS et al., 2006; HSU et al., 2006; HUANG et al., 2005; WIGGE et al., 2005; YOO et al., 2004; YOO et al., 2005). Improvements regarding the reduction of the long juvenile period in forest tree species and other woody plants seems to be possible in the near future.

The stability of transgene expression has a decisive influence on the efficiency of strategies for biological confinement of transgenic plants. Many studies on transgenic plants have shown that expression of transgenes is less stable than had originally been thought (HOENICKA and FLADUNG, 2006a, b). Most of these events reported fall into the class of homology-dependent gene silencing, which involves mechanisms that function at the level of transgene transcription or post-transcriptionally (reviewed in PASZKOWSKI, 1994; MEYER, 1995). Gene silencing has been reported in transgenic trees transformed with the *rolC* gene (FLADUNG, 1999; FLADUNG and KUMAR, 2002; KUMAR and FLADUNG, 2001; FLADUNG et al., 2004). Plants obtained in this study will be studied for several years under greenhouse condi-

tions in order to detect any change in the genetic expression of the sterility construct. This observations are important part of our biosafety research on transgenic forest tree species.

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